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# Deletion of the epidermal growth factor receptor in renal proximal tubule epithelial cells delays recovery from acute kidney injury

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To determine the role of epidermal growth factor receptor (EGFR) activation in renal functional and structural recovery from acute kidney injury (AKI), we generated mice with a specific EGFR deletion in the renal proximal tubule (EGFR<sup>ptKO</sup>). Ischemia–reperfusion injury markedly activated EGFR in control littermate mice; however, this was inhibited in either the knockout or wild-type mice given erlotinib, a specific EGFR tyrosine kinase inhibitor. Blood urea nitrogen and serum creatinine increased to a comparable level in EGFR<sup>ptKO</sup> and control mice 24 h after reperfusion, but the subsequent rate of renal function recovery was markedly slowed in the knockout mice. Twenty-four hours after reperfusion, both the knockout and the inhibitor-treated mice had a similar degree of histologic renal injury as control mice, but at day 6 there was minimal evidence of injury in the control mice while both EGFR<sup>ptKO</sup> and erlotinib-treated mice still had persistent proximal tubule dilation, epithelial simplification, and cast formation. Additionally, renal cell proliferation was delayed due to decreased ERK and Akt signaling. Thus, our studies provide both genetic and pharmacologic evidence that proximal tubule EGFR activation plays an important role in the recovery phase after acute kidney injury.

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KEYWORDS: acute kidney injury; epidermal growth factor; ischemia–reperfusion; proximal tubule

Acute kidney injury (AKI) occurs in 1% of hospital admissions, and up to 7% of hospitalized patients develop AKI.<sup>1</sup> At present, therapy for AKI is predominantly supportive, and the mortality remains unacceptably high.<sup>2</sup> Ischemia–reperfusion injury (I–R) is one of the most common causes of AKI, and the underlying pathogenesis involves the injury to nephron segments both from ischemia itself and from the inflammatory response.<sup>3</sup> The S3 segment of the proximal tubules is most vulnerable to I–R injury.<sup>3,4</sup> As a result of I–R injury, renal proximal tubule cells exhibit mitochondrial dysfunction, ATP depletion, impaired solute and ion transport, loss of cell polarity, and cytoskeletal disruption.<sup>5</sup>

The kidney has a remarkable capacity for regeneration, as evidenced by complete recovery of renal function following I–R injury. However, there remains controversy about the mechanisms underlying renal recovery from AKI. It is now considered unlikely that bone marrow–derived stem cells serve as the direct precursor for regenerating tubule epithelial cells,<sup>6–9</sup> although some studies have suggested that an interstitial, non-epithelial stem cell population might exist in adult kidney that could be recruited after injury to supply epithelial progenitors and contribute directly to nephron repair.<sup>10,11</sup> Studies in which renal epithelial cells were labeled using Cre-Lox technology have demonstrated that intrinsic epithelial cells are the major cells responsible for nephron repair in response to I–R injury.<sup>12</sup>

Locally produced growth factors that mediate proliferation and differentiation may have important roles in regeneration of renal epithelial cells following AKI.<sup>13</sup> In this regard, ligands for the EGF receptor (EGFR and ErbB1) have been shown to increase in response to a variety of experimental maneuvers that induce acute tubular injury, including I–R, mercuric chloride, aminoglycoside toxicity, and folic acid administration.<sup>14–16</sup> Similarly, EGFR expression and activation have been observed after acute renal injury.<sup>14,16–18</sup> Administration of exogenous EGF or other EGFR ligands has also been shown to accelerate recovery from ischemic or mercuric chloride–induced nephrotoxicity.<sup>19,20</sup> In our laboratory, previous studies indicated that functional EGFR activity is an essential component of the

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kidney's ability to recover from acute injury in response to mercuric chloride.<sup>21</sup>

To determine the role of renal proximal tubule EGFR activation and the mechanisms underlying renal functional and structural recovery from AKI, renal proximal tubule cell-specific EGFR deletion mice (EGFR<sup>ptKO</sup>) and wild-type control mice (EGFR<sup>fl/fl</sup>) were subjected to I-R injury. Our results indicate that both renal functional and structural recovery from I-R injury are delayed in EGFR<sup>ptKO</sup> mice compared with their littermate controls. In addition, we also found that the wild-type mice treated with the specific EGFR tyrosine kinase inhibitor, erlotinib (80 mg/kg by gavages), exhibited a markedly delayed rate of renal functional and structural recovery from I-R injury. Therefore, these studies provide both genetic and pharmacologic evidence that proximal tubule EGFR activation and subsequently induced EGFR-ERK and EGFR-PI3K-Akt signaling pathway activation have important roles in the recovery phase after AKI.

## RESULTS

### Phospho-EGFR is the most markedly upregulated receptor tyrosine kinase in response to renal I-R injury

To explore the potential receptor tyrosine kinases (RTKs) involved in kidney regeneration in response to I-R injury, we used a mouse phospho-RTK array to analyze the renal cortex tissue lysates after 2 days or 4 days reperfusion following bilateral kidney ischemia, and found that EGFR and ErbB2 were the most markedly phosphorylated kinases among the 39 different phosphorylated mouse RTKs included in the array (Supplementary Figure S1 online) in response to I-R injury within 48 h (Figure 1a and b). The only other positive signals detected indicated minimal phosphorylation of platelet-derived growth factor receptor alpha (PDGF-R $\alpha$ ) and macrophage-stimulating protein receptor (MSPR). After 4 days reperfusion, increased EGFR and ErbB2 activation was

still present, albeit at a lower level, and no new tyrosine kinase receptor activation was detected (data not shown). These data indicate EGFR and ErbB2 to be the RTKs most apparently activated during the renal I-R injury repair process.

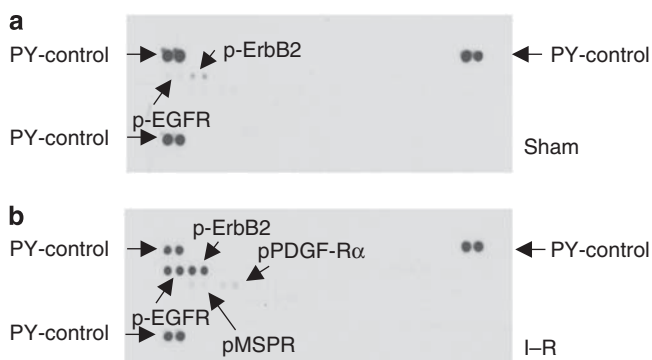
### Pharmacological EGF RTK inhibitor, erlotinib, markedly delayed renal functional and morphologic recovery after I-R injury

As an initial evaluation of the role of phosphorylation of EGF RTK in regeneration following I-R injury, we treated mice with erlotinib, an aquinazoline-based agent that competes with adenosine triphosphate for binding with the intracellular catalytic domain of epidermal growth factor receptor (HER1/EGFR) tyrosine kinase, inhibiting phosphorylation.<sup>22</sup> We found that EGFR phosphorylation increased after I-R injury and was inhibited by erlotinib. In addition, erlotinib markedly blunted phosphorylation of p44/p42 ERKs and Akt in response to I-R injury as shown in Figure 2a and b. These data confirmed that activation of p44/p42 ERKs and Akt in response to I-R injury are downstream of EGF receptor phosphorylation.

Erlotinib treatment significantly blocked the functional and structural recovery from I-R injury, although it did not alter the initial renal functional impairment and structural injury, as shown in Figure 3. To determine the effects of erlotinib on tubular epithelial cell proliferation after I-R injury, we performed Ki67 immunostaining. As shown in Figure 4, in vehicle-treated mice, Ki67 immunoreactivity peaked at 48 h, and very few proliferating cells were detected 6 days after reperfusion. However, erlotinib treatment significantly decreased the number of positive staining cells at 48 h after reperfusion and delayed cell proliferation.

### Selective deletion of EGFR in renal proximal tubules delayed renal structural and functional recovery from I-R injury

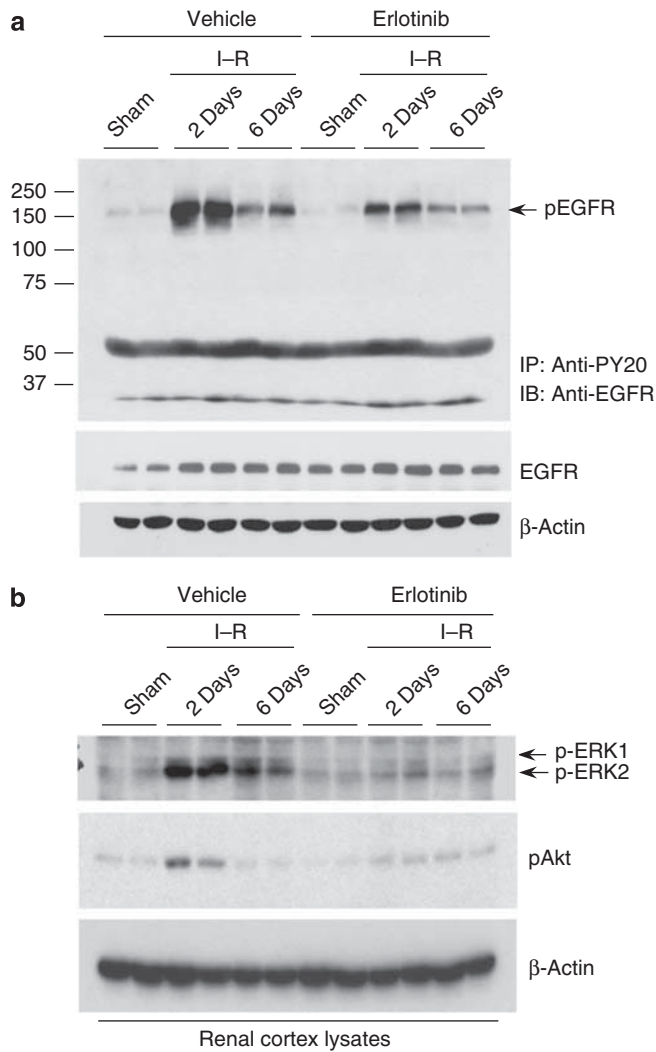
To determine the specific role of renal proximal tubule epithelial cell EGFR in kidney tubular epithelia cell regeneration after I-R injury, we developed a renal proximal tubule-specific EGFR<sup>ptKO</sup> mouse by crossing EGFR<sup>fl/fl</sup> mice with the Cre recombinase driven by the  $\gamma$ -GT promoter mice (EGFR<sup>ptKO</sup>).<sup>23</sup> Phospho-RTK array analysis showed that the deletion of EGFR in proximal tubule cells markedly decreased EGFR phosphorylation without affecting increases in ErbB2 phosphorylation in response to I-R injury, as shown in Figure 5a and b. Immunoblotting confirmed a marked decrease in phospho-EGFR expression following I-R injury in the EGFR<sup>ptKO</sup> mice. Similarly, in the EGFR<sup>ptKO</sup> mice, activation of 44/p42 ERK and Akt were markedly inhibited compared with wild-type mice, Figure 5c and d. We found similar renal function impairment in EGFR<sup>ptKO</sup> and their littermate control mice 24 h after I-R injury. Blood urea nitrogen levels were 104  $\pm$  24 vs. 101  $\pm$  22 mg/dl and serum creatinine levels were 0.698  $\pm$  0.027 vs. 0.695  $\pm$  0.040 mg/dl ( $n = 5-6$ ). However, 6 days after reperfusion, both blood urea nitrogen and serum creatinine levels remained elevated in EGFR<sup>ptKO</sup> mice



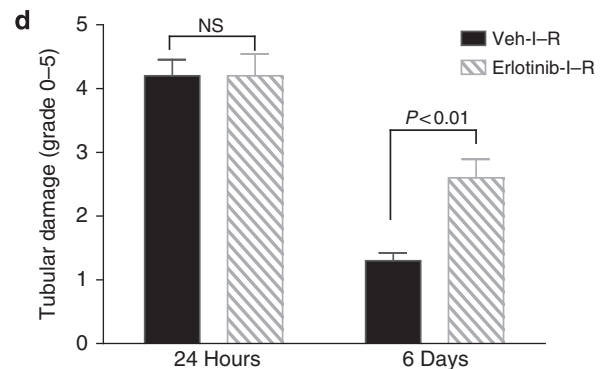
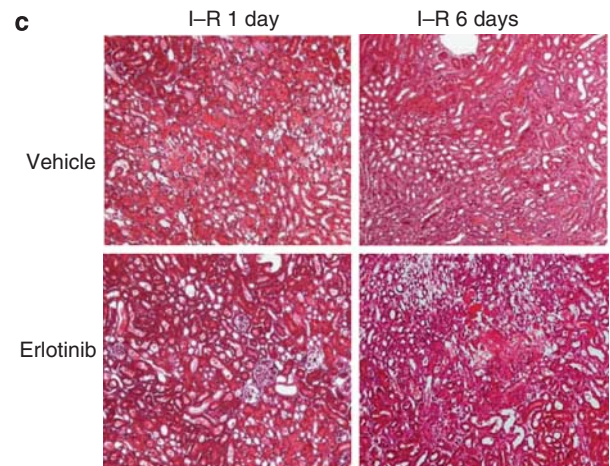
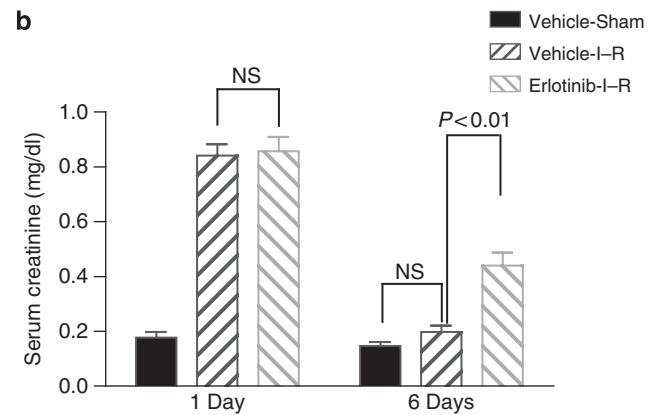
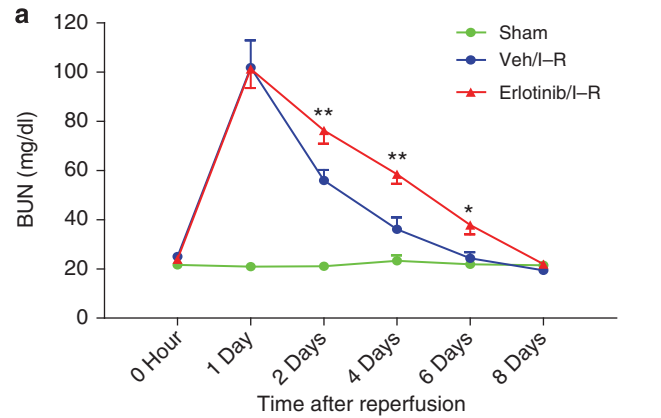
**Figure 1 | Phospho-epidermal growth factor receptor (EGFR) was the most markedly upregulated receptor tyrosine kinase (RTK) in response to renal ischemia-reperfusion (I-R) injury.**

Wild-type Balb/c mice were subjected to sham (a) or bilateral renal I-R injury (b). Renal cortex tissue lysates of five mice were pooled and analyzed by Mouse Phospho-Receptor Tyrosine Kinase (Phospho-RTK) Array 48 h after surgery. MSPR, macrophage-stimulating protein receptor; pPDGF-R $\alpha$ , phosphorylation of platelet-derived growth factor receptor alpha.

compared with their littermate control mice (blood urea nitrogen,  $57 \pm 11$  vs.  $22 \pm 1.5$  mg/dl,  $P < 0.0001$ ; serum creatinine,  $0.432 \pm 0.060$  vs.  $0.198 \pm 0.023$  mg/dl,  $P < 0.001$ ,  $n = 5-6$ ; Figure 6a and b). Histology indicated comparable

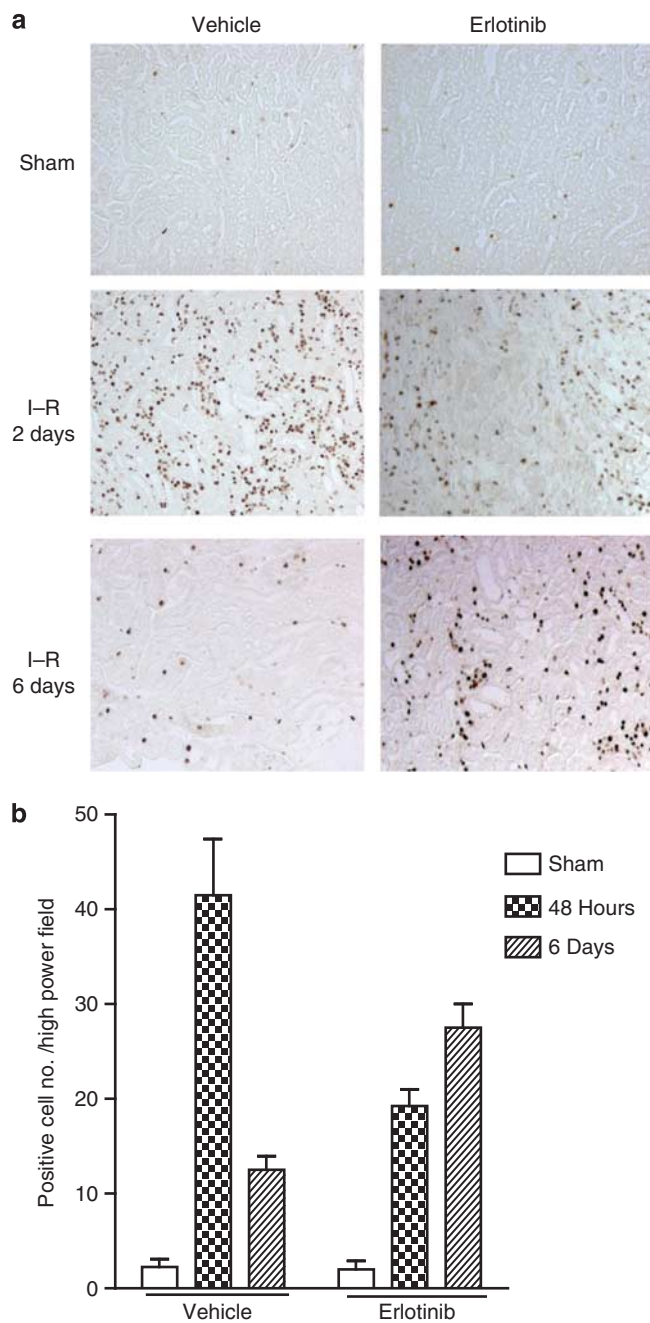


**Figure 2 | Administration of erlotinib inhibited activation of epidermal growth factor receptor (EGFR)-ERK and EGFR-Akt signaling pathways in response to ischemia-reperfusion (I-R) injury.** Balb/c mice were administered vehicle or erlotinib daily beginning 1 day before surgery, and renal cortex tissue lysates were prepared at different time points as indicated and subjected to immunoprecipitation (IP; **a**) or immunoblotting (IB; **a** and **b**) analysis by using indicated antibodies. Representative data of 5-6 separate experiments.



**Figure 3 | Erlotinib administration delayed renal structural and functional recovery from ischemia-reperfusion (I-R) injury.** Balb/c mice were administered vehicle or erlotinib daily beginning 1 day before surgery, and blood urea nitrogen (BUN; **a**) and serum creatinine (**b**) were measured at different time points after I-R injury as indicated. Histology (original magnification  $\times 200$ ) is indicated for days 1 and 6 after I-R injury (**c**), and tubular damages were scored as indicated in Materials and Methods (**d**). Values are means  $\pm$  s.e.m. ( $n = 6-8$  for each group).  $**P < 0.001$ ,  $*P < 0.05$ . NS, nonsignificant.

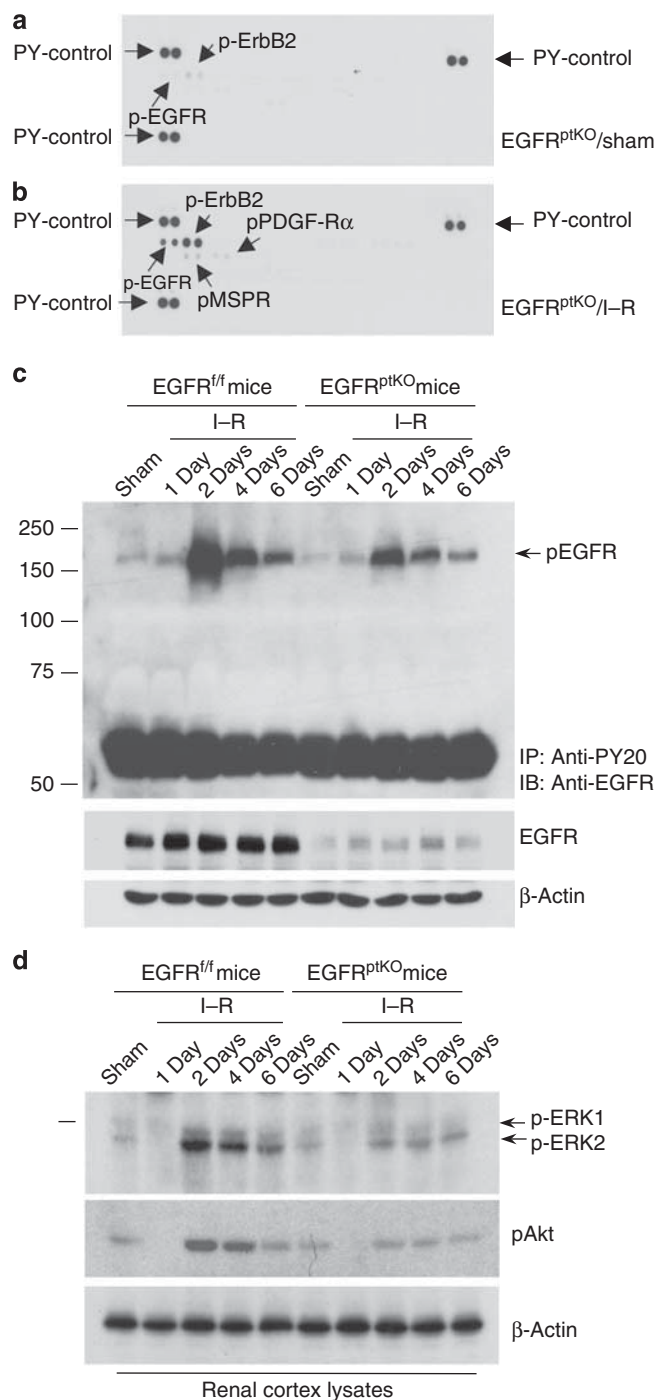




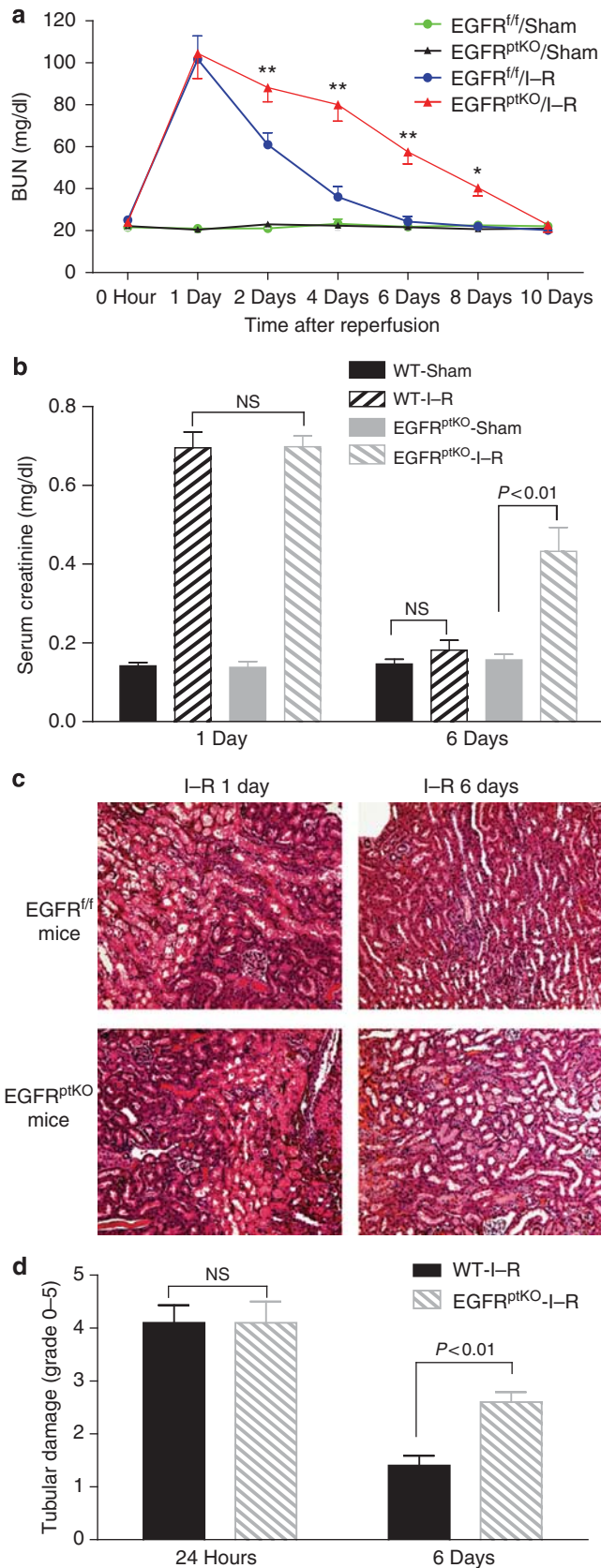
**Figure 4 | Erlotinib administration postponed the cell proliferation peak after ischemia-reperfusion (I-R) injury.** Mice were treated as indicated in Figure 2, and kidney sections of days 2 and 6 after I-R injury were analyzed by immunohistochemistry with antibodies against Ki67 (a), and positive staining cells were counted in each (original magnification  $\times 400$ ) field (b). Values are means  $\pm$  s.e.m ( $n = 5-8$  for each group).

injury at 24 h; however, there was minimal residual injury in littermate controls, whereas EGFR<sup>ptKO</sup> mice had persistent proximal tubule dilation and epithelial simplification and cast formation at day 6, as shown in Figure 6c and d.

I-R-induced AKI is characterized by early, alloantigen-independent inflammation, which may mediate injury.<sup>24-27</sup>



**Figure 5 | Renal proximal tubular epithelial cell epidermal growth factor receptor (EGFR) deletion attenuated increases in phospho-EGFR expression and activation of pEGFR-Akt pathways in response to ischemia-reperfusion (I-R) injury.** EGFR<sup>ptKO</sup> mice were subjected to sham or bilateral renal I-R injury. Renal cortex tissue lysates pooled from five mice were analyzed by Phospho-RTK Array 48 h after surgery (a and b). EGFR<sup>ptKO</sup> or EGFR<sup>+/+</sup> mouse renal cortex tissue lysates were prepared at different time points and subjected to immunoprecipitation (IP; c) or immunoblotting (IB; c and d) analysis with the indicated antibodies. MSPR, macrophage-stimulating protein receptor; pPDGF-R $\alpha$ , phosphorylation of platelet-derived growth factor receptor alpha.



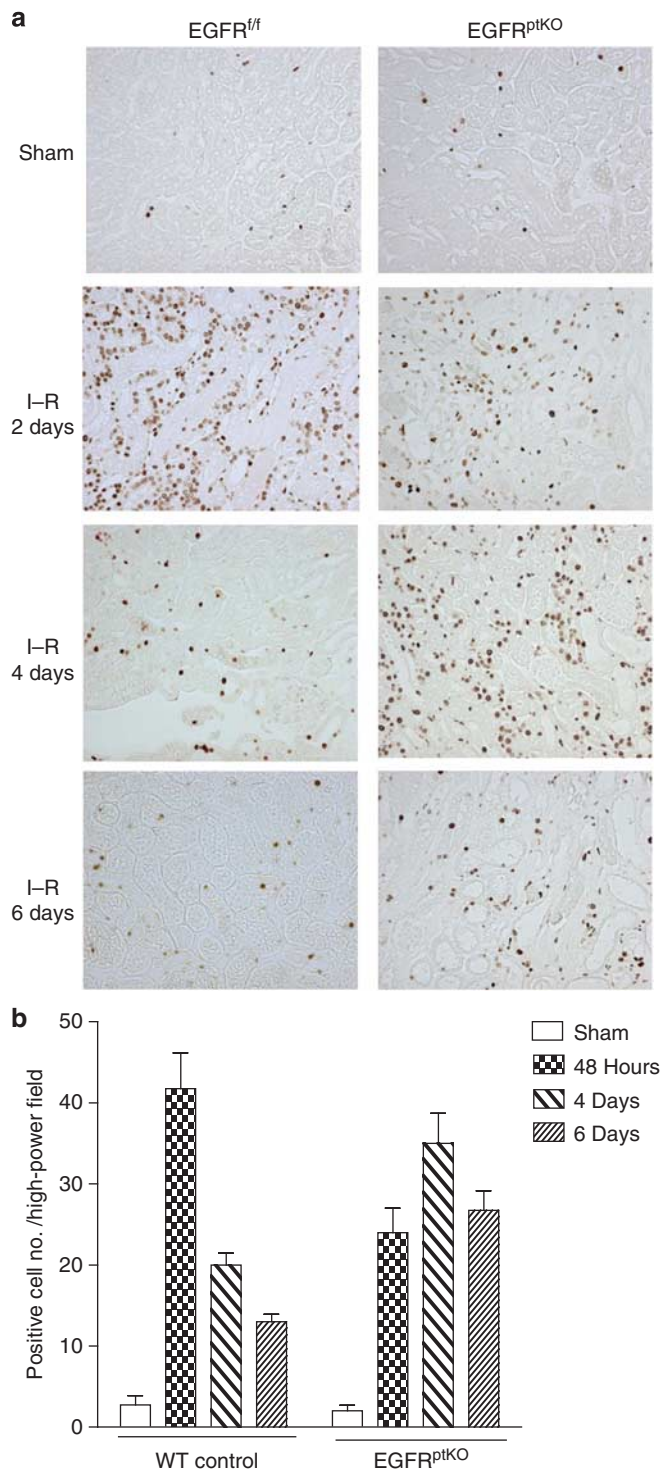
To understand whether proximal tubule EGFR was involved in I-R-induced infiltration of neutrophils or macrophages, we assayed the neutrophil marker, Gr-1, and macrophage marker, F4/80, and found that proximal tubule epithelial cell EGFR deletion did not affect infiltration of these cells in response to I-R injury (Supplementary Figures S2 and S3 online). However, Ki67 immunostaining indicated that proximal tubular epithelial cell EGFR deletion delayed cell proliferation after I-R injury (Figure 7). These results indicate that, in the proximal tubule, the EGF RTK phosphorylation cascade that activates the p44/p42 ERKs and Akt is an important mechanism for renal tubular cell regeneration in response to I-R injury.

**DISCUSSION**

Ischemia-reperfusion injury is one of the most common causes of AKI. Acute tubular necrosis and apoptosis, especially affecting the S3 segment of the proximal tubules and possibly the thick ascending limb, represent pathogenic consequences of I-R. Fortunately, the kidney has a remarkable capacity to regenerate, and increasing evidence indicates that intrinsic epithelial cells are responsible for nephron repair following acute renal injury.<sup>12</sup> Locally produced growth factors that mediate proliferation and differentiation may have important roles in kidney tubular cell regeneration from acute tubular necrosis.<sup>13</sup> Previous studies have shown that expression of EGFR ligands, HB-EGF and transforming growth factor- $\alpha$ , increase in response to AKI,<sup>14-16</sup> and administration of exogenous EGF or other EGFR ligands has also been shown to accelerate recovery.<sup>19,20</sup> Moreover, EGFR activation is essential for acute renal injury recovery.<sup>14,16-18,21</sup> Because of fetal or perinatal lethality, it is not possible to use global EGFR knockout mice in AKI studies, and thus in the current studies we generated proximal tubule cell-specific deletion of EGFR. We found that, compared with their wild-type littermates, EGFR<sup>ptKO</sup> mice exhibited markedly delayed functional and structural recovery from I-R injury. In addition, administration of a specific EGFR tyrosine kinase inhibitor, erlotinib, also markedly delayed renal functional and structural recovery.

Although innate immune system activation and infiltration of inflammatory cells into the injured kidney is an important pathogenic mechanism of AKI,<sup>28</sup> there was no difference in neutrophil or macrophage infiltration in wild-type and EGFR<sup>ptKO</sup> mice in response to I-R injury. This

**Figure 6 | Selective deletion of epidermal growth factor receptor (EGFR) in renal proximal tubules delayed renal structural and functional recovery from ischemia-reperfusion (I-R) injury.** Following I-R injury to EGFR<sup>ptKO</sup> or EGFR<sup>fl/fl</sup> mouse, blood urea nitrogen (BUN; **a**) and serum creatinine (**b**) were measured, and histology (original magnification,  $\times 200$ ) at day 1 and day 6 after I-R injury are presented (**c**), and tubular damages were scored as indicated in Materials and Methods (**d**). Values are means  $\pm$  s.e.m. ( $n = 6-8$  for each group). \*\* $P < 0.001$ , \* $P < 0.05$ . NS, nonsignificant; WT, wild-type mice.



**Figure 7 | Renal proximal tubular epithelial cell epidermal growth factor receptor (EGFR) deletion delayed cell proliferation after ischemia-reperfusion (I-R) injury.** Ki67 expression in EGFR<sup>ptKO</sup> or EGFR<sup>fl/fl</sup> mouse kidneys was performed at days 2, 4, and 6 after I-R injury (a), and the positive staining cells were counted in each (original magnification  $\times 400$ ) field (b). Values are means  $\pm$  s.e.m. ( $n = 5-8$  for each group).

finding is consistent with the apparent lack of difference from wild type in the initial injury seen in the EGFR<sup>ptKO</sup> mice or in mice treated with erlotinib.

In contrast, the rate of recovery from I-R injury was markedly delayed with EGFR inhibition. In this regard, it was striking that, of the 39 RTKs included on the array, the dominant signals were EGFR and the associated receptor, ErbB2. ErbB2 is not itself activated by ligands, but heterodimerizes with EGFR and other ErbB receptor family members. Migration, proliferation, and repair of physiological functions are the three crucial processes that must be achieved for structural and functional regeneration of the nephron.<sup>5</sup> PI3K-Akt and MAPK (ERK1/2) are two major downstream effectors of EGFR activation, and it is known that PI3K-Akt activation is closely related to cell survival and migration,<sup>29</sup> and that ERK1/2 signaling pathway activation triggers cell proliferation.<sup>30,31</sup> In our study, we observed activation of both PI3K-Akt and ERK1/2 signaling pathways in response to I-R injury, and activation of both pathways was markedly attenuated by either proximal tubule EGFR deletion or administration of an EGFR tyrosine kinase inhibitor. These findings suggest that EGFR-induced PI3K-Akt and p44/p42 ERKs signaling are major mechanisms for the regeneration of renal tubules in response to I-R injury. Although the mechanisms of EGFR tyrosine kinase activation in response to I-R injury need to be studied further, this study provides both genetic and pharmacologic evidence that proximal tubule EGFR activation has an important role in the renal functional and morphological recovery after AKI.

## MATERIALS AND METHODS

### Reagents and antibodies

Erlotinib was purchased from LC Laboratories (Woburn, MA). Antibodies against EGFR, phospho-ERK, and phosphoAkt were from Cell Signaling Technology (Beverly, MA). Antibody against Ki-67 was from Vector Laboratories (Burlingame, CA), and antibody against  $\beta$ -actin and all secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Rat anti-mouse F4/80 and Gr-1 antibodies were from AbD Serotec (Raleigh, NC). The Mouse Phospho-Receptor Tyrosine Kinase (Phospho-RTK) Arrays were purchased from R&D Systems (Minneapolis, MN).

### Generation of EGFR<sup>ptKO</sup> null mice

All experiments were approved by the Vanderbilt University Institutional Animal Use and Care Committee, and all experiments were conducted in accordance with National Institutes of Health guidelines. EGFR floxed (EGFR<sup>fl/fl</sup>) mice were generated by flanking exons 3 of the *EGFR* gene with two LoxP sites, as we have previously described,<sup>32</sup> and back-crossing onto a BALB/c background for 10 generations. These EGFR<sup>fl/fl</sup> mice were crossed with transgenic mice carrying Cre recombinase under the control of the  $\gamma$ -glutamyl transpeptidase promoter ( $\gamma$ -GT-Cre<sup>+</sup>).<sup>33</sup> The  $\gamma$ -GT promoter has been used to successfully target different genes of interest primarily to the renal proximal tubule.<sup>33</sup> Age-matched EGFR<sup>fl/fl</sup> littermates lacking  $\gamma$ -GT-Cre transgene were used as wild-type controls for EGFR<sup>ptKO</sup> mice lacking the EGFR along the proximal tubule. Mice were genotyped by PCR with the following primers: 5-CTTTGGA GAACCTGCAGATC-3 and 5-CTGCTACTGGCTCAAGTTTC-3 for verification of the EGFR gene floxed mice, 5-ACACTAGCACTGAC TGCTGG-3 and 5-CTGCTACTGGCTCAAGTTTC-3 for verification of the EGFR wild-type mice, and 5-AGGTGTAGAGAAGGCACCT



AGC-3 and 5-CTAATCGCCATCTTCCAGCAGG-3 for verification of the  $\gamma$ -GT-Cre expression.

### I-R surgery

Nine- to ten-week-old EGFR<sup>ptKO</sup> mice and their littermates or wild-type Balb/c mice (Jackson Laboratory, Bar Harbor, ME) were anesthetized by peritoneal injection of 50 mg/kg of nembutal sodium. Bilateral renal pedicles were exposed using a midline ventral incision and then clamped for 35 min followed by reperfusion, which was verified by visual inspection of the kidneys. Sham animals had an incision plus 35 min of waiting time without clamping. After ischemia or sham treatment, muscle and skin layers were sutured, respectively. A volume of 0.8 ml of pre-warmed 0.9% saline solution was administered to each mouse right before closing the incision, in order to prevent dehydration.

### Renal function measurement

Mouse blood samples were collected at 1, 2, 4, 6, 8, and 10 days after surgery, and blood urea nitrogen levels were measured as previously described.<sup>34</sup> Serum creatinine was measured by high-performance liquid chromatography.

### Histological examination

Kidneys embedded in paraffin were sectioned at 5  $\mu$ m and stained with hematoxylin and eosin by standard methods. Tubular damage was scored by calculation of the percentage of tubules in the cortico-medullary junction that displayed cell necrosis, loss of the brush border, cast formation, and tubular dilatation as follows: 0, none; 1,  $\leq$  10%; 2, 11–25%; 3, 26–45%; 4, 46–75%; and 5, > 76%.<sup>35</sup> At least 10 high-power fields (original magnification  $\times$  200) per section for each sample were examined,  $n = 5-8$ .

### Immunoprecipitation and immunoblotting

Procedures were performed as we previously described.<sup>36,37</sup> Briefly, EGFR<sup>ptKO</sup> mice and their littermates or wild-type Balb/c mice were killed at 1, 2, 4, and 6 days after surgery, and mouse kidney cortices were dissected and homogenized in RIPA buffer at different time points after reperfusion. After centrifugation at 10,000g for 15 min at 4 °C, equal amounts of protein lysate were loaded directly or after immunoprecipitation onto 7 to 12% SDS-polyacrylamide gel electrophoresis, transferred onto Immobilon-P transfer membranes (Millipore, Bedford, MA), and probed with the indicated primary antibody. The primary antibodies were detected with peroxidase-labeled goat anti-rabbit immunoglobulin-G or goat anti-mouse immunoglobulin-G and exposed on film by using enhanced chemiluminescence (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK).

### Receptor tyrosine kinase array analysis

At 48 h or 4 days after reperfusion, EGFR<sup>ptKO</sup> and their littermates' kidney cortex lysates were prepared in NP-40 Lysis Buffer (1% NP-40, 20 mmol/l Tris-HCl (pH 8.0), 137 mmol/l NaCl, 10% glycerol, 2 mmol/l EDTA, 1 mmol/l sodium orthovanadate, 10  $\mu$ g/ml Aprotinin, 10  $\mu$ g/ml Leupeptin, and 10  $\mu$ g/ml Pepstatin). For each group, renal cortical tissue lysates were pooled from five different animals, and the same amount of proteins were used for analysis, following the instruction for the Mouse Phospho-RTK Array Kit (R&D Systems, Minneapolis, MN).

### Immunohistochemistry

Mice were killed at different time points after I-R injury, and kidneys were harvested followed by overnight fixation in 4%

paraformaldehyde. The fixed kidneys were dehydrated through a graded series of ethanol, embedded in paraffin, sectioned (5  $\mu$ m), and mounted on glass slides. After deparaffinization and rehydration, antigen retrieval was performed using Antigen Unmasking Solution (Vector Laboratories). The sections were blocked with 2.5% normal goat serum and then incubated with primary antibodies against Ki67 (at 1:400), against F4/80 (at 1:2000), or Gr1 (at 1:100) and incubated overnight at 4 °C. The sections were washed three times in phosphate-buffered saline followed by using VECTASTAIN ABC kits (Vector Laboratories) following the manufacturer's instruction. The sections were counterstained with hematoxylin. Images were captured using an AxioCam HRc digital camera (Carl Zeiss, Thornwood, NY), and the positive staining cells were counted in each (original magnification  $\times$  400) field, i.e., 10 randomly chosen fields for each section. Values are means  $\pm$  s.e.m. ( $n = 5-8$  for each group).

### Statistics

Data are presented as means  $\pm$  standard error of the mean for separate experiments. An unpaired Student's *t*-test was used for statistical analysis, and for multiple group comparisons analysis of variance and Bonferroni *t*-tests were used; a *P*-value of <0.05 compared with control was considered statistically significant.

### DISCLOSURE

All the authors declared no competing interests.

### ACKNOWLEDGMENTS

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### SUPPLEMENTARY MATERIAL

**Figure S1.** Mouse Phospho-receptor Tyrosine Kinases Array coordinates.

**Figure S2.** Renal proximal tubular epithelia cell EGFR deletion did not alter infiltration of neutrophils in kidney in response to I-R injury.

**Figure S3.** Renal proximal tubular epithelia cell EGFR deletion did not affect infiltration of macrophages in kidney in response to I-R injury. Supplementary material is linked to the online version of the paper at <http://www.nature.com/ki>

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